Science

Type, Density, and Location of Immune Cells Within Human Colorectal Tumors Predict Clinical Outcome

Volume 313(5795), 29 September 2006, pp 1960-1964

[Reports: Cancer]

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Supporting Online Material: www.sciencemag.org/cgi/content/full/313/5795/1960/DC1 ; Materials and Methods; Figs. S1 to S10; Tables S1 to S12

25 April 2006; accepted 30 August 2006

10.1126/science.1129139

Outline

Graphics

Abstract^

The role of the adaptive immune response in controlling the growth and recurrence of human tumors has been controversial. We characterized the tumor-infiltrating immune cells in large cohorts of human colorectal cancers by gene expression profiling and in situ immunohistochemical staining. Collectively, the immunological data (the type, density, and location of immune cells within the

tumor samples) were found to be a better predictor of patient survival than the histopathological methods currently used to stage colorectal cancer. The results were validated in two additional patient populations. These data support the hypothesis that the adaptive immune response influences the behavior of human tumors. In situ analysis of tumor-infiltrating immune cells may therefore be a valuable prognostic tool in the treatment of colorectal cancer and possibly other malignancies.

Tumors in mice and humans often contain infiltrates of immune cells. Experiments with immune-deficient mice have provided data supporting the role of adaptive immunity in cancer immunosurveillance (1-4). Tumor cells can express antigens and become targets for a T cell-mediated adaptive immune response (5, 6). The differentiation of naïve $CD4^+$ T cells into T helper type 1 (T_H1) cells producing interferon gamma (IFN-[gamma]) promotes CD8 T cell-mediated adaptive immunity (7). In mice, immune cells appear to prevent the development of tumors and inhibit tumor progression (1, 3, 4). Anti-tumor immunity also leads to immunoediting, a process favoring the eventual outgrowth of tumor cells with reduced immunogenicity (3).

The role of immune cells in human neoplasia is less clear (8). Immune cells can release inflammatory mediators with proangiogenic and prometastatic effects (9-14). Tumor-infiltrating lymphocytes in melanoma (15), colorectal cancers (CRCs) (16-18), and ovarian cancers (19, 20) have been shown to inhibit tumor growth and are associated with improved prognoses. After antigen stimulation, a small population of antigen-specific memory T cells remains in the tissues (21). We recently showed that human CRCs with a high density of infiltrating memory and effector memory T cells were less likely to disseminate to lymphovascular and perineural structures and to regional lymph nodes (22). Using the same cohort of patients, we investigated the relationship between the type, density, and location of immune cells within tumors and the clinical outcome of the patients.

To this end, we conducted genomic and in situ immunostaining analyses on tumors from 75 and 415 patients, respectively (table S1). The data were entered into a dedicated Tumoral MicroEnvironment Database (TME.db; access available upon request). We used quantitative real-time polymerase chain reaction to evaluate the expression levels of genes related to inflammation, T_H1 adaptive immunity, and immunosuppression. These genes showed variable expression patterns in the 75 tumors studied (fig. S1). Correlation analyses performed

between all genes showed 39 highly significant combinations ($P < 0.0001$) (fig. S1 and table S2). We identified a dominant cluster of comodulated genes for T_H1 adaptive immunity [genes encoding T-box transcription factor 21, interferon regulatory factor 1, IFN-[gamma], CD3-[zeta], CD8, granulysin, and granzyme B (GZMB)] (Fig. 1A). A hierarchical tree structure classifying the patients according to the expression levels of genes from this cluster revealed an inverse correlation between the expression of these genes and tumor recurrence (*P* value comparing patient groups, all $P < 0.05$) (Fig. 1B). These data suggest that T_H1 adaptive immunity has a beneficial effect on clinical outcome.

Fig. 1. (**A**) Correlation analyses performed between the 18 immunogenes were uploaded into the Genesis clustering program (28-30). A correlation matrix followed by unsupervised hierarchical clustering (Pearson uncentered algorithm) is represented from $R = -0.8$ negative correlation (green) to $R = 0.8$ positive correlation (red). For all correlations with $0.4 < R < 0.9$, $P < 0.05$ (table S1). The correlation matrix reveals a dominant cluster of co-modulated genes for T_H1 adaptive immunity and two clusters of genes encoding mediators of inflammation and immunosuppression. (**B**) Hierarchical tree structure classifying the 75 patients according to the mRNA levels of the seven genes from the T_H1 adaptive cluster, from maximal (red) to minimal (blue) expression levels. The percentage of patients with tumor recurrence (relapse) is indicated. Patients with a homogeneous increased expression of genes for T_H1 adaptive immunity had the best prognosis. Log-rank tests comparing the disease-free survival times between patient groups reached statistical significance ($P < 0.05$ for numbers 1, 2, and 3). In contrast, expression levels of inflammatory and immunosuppressive genes showed no correlation with tumor recurrence. MMP-7, matrix metalloproteinase 7; PTSG2, prostaglandin-endoperoxide synthase 2; IL8, interleukin-8; BIRC5, baculoviral IAP repeat-containing 5 (survivin); CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; GLNY, granulysin; IRF1, interferon regulatory factor 1; TBX, T-box 21 (TBET); TNFRSF10A, tumor necrosis factor receptor superfamily, member 10a (TRAILR1); TGFB1, transforming growth factor-[beta] 1; VEGF, vascular endothelial growth factor.

We next used tissue microarrays to investigate the in situ adaptive immune response in the center of the tumor (CT) and the invasive margin (IM) of 415 CRCs. Immunostainings for total T lymphocytes (CD3), CD8 T cell effectors and their associated cytotoxic molecule (GZMB), and memory T cells (CD45RO) were quantified with the use of a dedicated image analysis work-station (Fig. 2A and figs.

S2 to S4). Tumors from patients without recurrence had higher immune cell densities (CD3, CD8, GZMB, and CD45RO) within each tumor region (CT and IM), than did those from patients whose tumors had recurred (Fig. 2B). In each tumor region (CT and IM) and for each marker (CD3, CD8, GZMB, and CD45RO), there was a statistically significant correlation between immune cell density and patient outcome for a large range of cutoff values (fig. S5). In particular, using the cutoff that yielded the minimum *P* value for disease-free survival, the densities of $CD3^+$, $CD8^+$, $GZMB^+$, and $CD45RO^+$ cells in each tumor region (CT and IM) allowed the stratification of patients into groups with different disease-free survival rates [*P* values corrected after (23), ranging from 1.0×10^{-2} to 4.8×10^{-6}] and overall survival rates (*P* values ranging from 5.5×10^{-3} to 7.9×10^{-8}) (Fig. 2C and tables S3 and S4). Reanalyses of the data using 100 repetitions of twofold cross-validations after (24) (tables S3 and S4) or setting the cutoff at the median of the data sets (tables S5 and S6) provided concordant results as to the prognostic value of each immune parameter.

Fig. 2. (**A**) (Left) A representative example of CD3 immunostaining of a CRC tissue microarray (top). $CD3^+$ T cells (brown) and tumor cells (blue) are shown. (Right) Digital image analyzed with the image software SpotBrowser, with tissue represented in yellow and $CD3^+$ cells represented in red. The densities of adaptive immune cells ($CD3^+$, $CD8^+$, $GZMB^+$, and $CD45RO^+$ cells) were recorded as the number of positive cells per unit of tissue surface area. (**B**) Comparison of the mean (±SE) of immune cell densities in the CT and IM from patients with tumor recurrence (black bars) or without tumor recurrence recurrence (white bars). (**C**) Overall survival time for all patients, accounting for censoring (75th percentile), with high densities (red bars) or low densities (black bars) or adaptive immune cells in each tumor region (CT or IM). (**D** to **F**) Three independent cohorts of CRC patients were analyzed in a blinded manner for $CD3_{CT}/CD3_{IM}$ patterns [(D), n = 415; (E), n = 119; (F), n = 69 patients]. Kaplan-Meier curves illustrate the duration of disease-free survival according to the $CD3^+$ cell density in a single tumor region in the CT (left panels) or IM (middle panels) and in both tumor regions (right panels). In each cohort, for each tumor region, high (Hi) and low (Lo) CD3 densities were plotted according to the cutoff value of $CD3^+$ cell density defined at the median of the cohort (50% of patients with high cell density and 50% of patients with low cell density). In single-region analysis (left and middle panels), red lines indicate $CD3^{Hi}$ and black lines indicate $CD3^{Lo}$. In combined analysis (right panels), red lines indicate $CD3_{CT}{}^{Hi}CD3_{IM}{}^{Hi}$, black lines indicate $CD3_{CT}{}^{Lo}CD3_{IM}{}^{Lo}$, and blue lines indicate heterogeneous CD3 densities with $CD3_{CT}{}^{Lo}$

plus $CD3_{IM}^{Hi}$ or $CD3_{CT}^{Hi}$ plus $CD3_{IM}^{Lo}$ ($CD3_{CTIM}^{Het}$).

We investigated whether the combined analysis of tumor regions could improve the prediction of patient survival. For all the markers of adaptive immunity (CD3, CD8, GZMB, and CD45RO), the combined analysis of CT plus IM regions [high density in both regions (HiHi) versus low density in both regions (LoLo)] increased the accuracy of prediction of disease-free and overall survival time for the different patient groups, as compared to single-region analysis (Hi versus Lo) (Fig. 2, D to F; figs. S6 and S7; and tables S3 to S6). Data were also analyzed using twofold cross-validation after (24) (100 repetitions for each marker), showing highly significant differences (tables S3 and S4). $CD3_{CT}/CD3_{IM}$ density was associated with the smallest *P* values for disease-free and overall survival analyses ($P = 7.6 \times 10^{-8}$ and $P = 4.0 \times 10^{-7}$, respectively) (tables S3 and S4). To confirm these results, we analyzed an additional cohort of patients who were different from those in the first series and a third cohort of CRC patients from another hospital. For each cohort, we determined the median cutoff values for $CD3_{CT}/CD3_{IM}$ density (50% of patients with a high density and 50% of patients with a low density). The two independent cohorts (Fig. 2, E and F) confirmed the data obtained on the first series (Fig. 2D). All statistical analyses were also performed for the subgroup of patients without concomitant distant metastasis [Union Internationale Centre le Cancer-Tumor Node Metastasis (UICC-TNM) cancer stages I, II, and III]. Significant *P* values were observed for $CD3_{CT}/CD3_{IM}$, $CD8_{CT}/CD8_{IM}$, and $CD45RO_{CT}/CD45RO_{IM}$ densities for predicting disease-free survival and overall survival (figs. S8 and S9 and tables S7 to S10).

We determined whether these immune criteria could discriminate patient outcome at each step of cancer progression. Patients were stratified according to the UICC-TNM classification (25) (Fig. 3A). A strong in situ immune reaction in both tumor regions correlated with a favorable prognosis regardless of the local extent of the tumor and of invasion of regional lymph nodes (stages I, II, and III). Conversely, a weak in situ immune reaction in both tumor regions correlated with a poor prognosis even in patients with minimal tumor invasion (stage I) (Fig. 3B). We recently demonstrated the importance of the density of $CD45RO^+$ memory T cells in limiting the tumor dissemination of CRCs (22). We found that patients with low densities of $CD3^+$ cells and $CD45RO^+$ memory T cells in both tumor regions (CT

and IM) had a very poor prognosis, similar to that of patients with concomitant distant metastasis (stage IV) (Fig. 3C). In multivariate analysis, after adjusting for tumor invasion (T stage), tumor differentiation, and lymph node invasion (N stage), $CD3_{CT}/CD3_{IM}$ density (HiHi, Heterogeneous, and LoLo) remained an independent prognostic factor, with the highest hazard ratio (HR) and the smallest *P* value in disease-free survival analysis [HR = 2.379; $P = 1.4 \times 10^{-6}$, corrected after (26)] (table S11). $CD3_{CT}/CD3_{IM}$ density was the only independent parameter associated with overall survival (HR = 1.89; $P = 1.2 \times 10^{-5}$) (table S12). The histopathological parameters were no longer associated with disease-free and overall survival in patients with coordinated high or low densities of the immune markers in both tumor regions (HiHi versus LoLo) (tables S11 and S12).

Fig. 3. (**A**) Kaplan-Meier curves illustrate the duration of disease-free survival according to the UICC-TNM stages [stage I, red line (*n* = 75 patients); stage II, green line (*n* = 137); stage III, blue line (*n* = 99), and stage IV, black line (*n* = 95)] in patients with CRCs. (**B**) Kaplan-Meier curves illustrate the duration of disease-free survival according to the UICC-TNM stages [as in (A)] and according to the density of $CD3^+$ cells in combined tumor regions ($CD3_{CT}^{Lo}CD3_{IM}^{Lo}$, thick lines, *n* = 93 patients; $CD3_{CT}^{Hi}CD3_{IM}^{Hi}$, thin lines, *n* = 109). The subgroup of patients that did not appear to have a coordinated in situ immune reaction in tumor regions (Hi/Lo or Lo/Hi for $CD3^+$ cell densities) presented similar Kaplan-Meier curves as the entire cohort (fig. S10). (**C**) Kaplan-Meier curves illustrate the duration of disease-free survival according to the UICC-TNM stages and to the density of $CD3^+$ and $CD45RO^+$ cells in combined tumor regions ($CD3_{CT}^{Lo}CD3_{IM}^{Lo}$ plus $CD45RO_{CT}^{Lo}CD45RO_{IM}^{Lo}$, thick lines, *n* = 16 patients; $CD3_{CT}^{Hi}CD3_{IM}^{Hi}$ plus $CD45RO_{CT}^{Hi}CD45RO_{IM}^{Hi}$, thin lines, *n* = 88). Cutoff values were 250, 640, 60, and 190 for $CD3_{CT}$, $CD3_{IM}$, $CD45RO_{CT}$, and $CD45RO_{IM}$, respectively. In (B) and (C), log-rank statistical test, ** $P < 10^{-4}$; ns, not significant.

Our results suggest that once human CRCs become clinically detectable, the adaptive immune response plays a role in preventing tumor recurrence. Despite immunoediting (2), the beneficial effect of the adaptive immunity may persist throughout tumor progression (stages II and III). Intratumoral T cells could modify tumor stroma or tumor cells in ways that attenuate the metastatic potential of tumor cells. We found a positive correlation between the presence of markers for T_H1 polarization and of cytotoxic and memory T cells and a low incidence of

tumor recurrence. This argues for immune-mediated rejection of persistent tumor cells after surgery. We hypothesize that the trafficking properties and long-lasting anti-tumor capacity of memory T cells (27) play a central role in the control of tumor recurrence.

The type, density, and location of immune cells in CRCs had a prognostic value that was superior to and independent of those of the UICC-TNM classification (25). This suggests that time to recurrence and overall survival time are governed in large part by the state of the local adaptive immune response. The immunological criteria that we have used may lead to revision of the current indicators of clinical outcome and may help identify the high-risk patients who would benefit most from adjuvant therapy. Finally, this approach may be useful for the investigation of other tumor types.

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31. We thank A. Rodi and M. Pelegrin for expert technical assistance; I. Gresser for

helpful comments and critical review of the manuscript; and D. Frucht, C. Anderson, and T. Pokrovska for critically reading the manuscript. Supported by the Association pour la Recherche sur le Cancer (ARC) through the Alliance pour la Recherche sur le Cancer network (ARECA), INSERM, Action Concertée Incitative ACI IMPBio, Université Paris-Descartes Paris 5, and the Austrian Federal Ministry of Education, Science and Culture (GBN-AU project Bioinformatics Integration Network).

Accession Number: 00007529-200609290-00020